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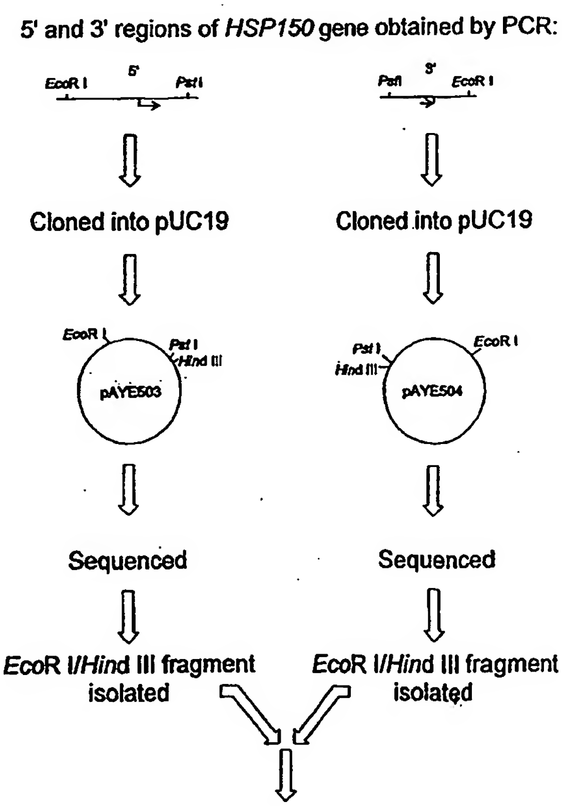
(54) Title: YEAST STRAINS

(57) Abstract

Reduction (preferably elimination) of the HSP150 protein in a yeast used to produce desired foreign proteins, especially human albumin, facilitates subsequent purification of the protein.



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YEAST STRAINS

Field of the Invention

The present invention relates to the production of heterologous proteins by yeast species and more particularly to an adaptation of the yeast in which the protein is produced.

Background and Prior Art

In recent years, yeasts have been widely used as host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992), including recombinant human albumin (rHA) (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for the production of secreted as well as cytosolic proteins.

When yeasts are utilised to produce a desired heterologous protein by secretion into the growth medium, a large number of host-derived proteins may also be present, including other proteins secreted by the host but also intracellular proteins present in the supernatant as the result of leakage from cells or cell lysis. In processes in which the protein is not secreted, there is of course an even higher level of contamination with intracellular yeast proteins. It is necessary to purify the desired protein and to remove these contaminating proteins from the preparation; such methods have been disclosed in WO 92/04367 and EP 524 681. The majority of contaminating proteins will have physicochemical properties sufficiently different from the desired protein to permit efficient separation by standard techniques, such as ion exchange or size exclusion chromatography. The prior art gives the impression that such proteins can be satisfactorily removed by such techniques; see, for example EP

524 681 (Gist-brocades), EP 570 916 (Green Cross) and EP 464 590 (Green Cross). Indeed, we have developed sophisticated chromatographic techniques (unpublished) to remove contaminating proteins from desired proteins.

Summary of the Invention

We have now also adopted a different approach and have identified the gene responsible for a protein, namely the *HSP150* gene, which co-purifies with recombinant human albumin (rHA) and, in principle, with other desired proteins. In accordance with the invention, we eliminate the contaminating protein from the initial fermentation, rather than develop highly sophisticated and complex means of removal during purification. This protein was not previously known to be a co-purifying contaminant.

In one aspect of the invention, the *HSP150* gene is functionally deleted from the genome of the host. This has not caused any detrimental effects on production of the desired protein and removes a potential contaminant that has proven difficult to remove by standard purification techniques. Despite the presence of at least two closely related genes encoding proteins very similar to Hsp150, *PIR1* and *PIR3*, in such modified yeast, rHA purified from these organisms does not contain detectable levels of any protein from this family.

The *S. cerevisiae* Hsp150 protein was originally described by Russo *et al* (1992) and was shown to be produced constitutively, to be extensively O-glycosylated and to be secreted efficiently into the growth medium. A 7-fold increase in the level of Hsp150 protein was seen when cells grown at 28°C were shifted to 37°C. Makarow has proposed preparing fusions of Hsp150 (or fragments thereof) and a desired protein, in order to achieve enhanced, controllable secretion (WO 93/18167). The *HSP150* gene encodes a primary translation product of 413 amino acids, including an N-terminal secretion signal

sequence of 18 amino acids that is not present in the mature protein. A further post-translational processing event occurs C-terminal to a pair of basic residues to yield two subunits of 54 and 341 amino acids which remain associated. The 341 amino acid subunit contains 11 tandem repeats of a 19 amino acid sequence, the function of which is unknown. Homologues of the *HSP150* gene were found in *Torulaspora delbrueckii, Kluyveromyces marxianus* and *Schizosaccharomyces pombe* (Russo *et al*, 1992).

The same protein has been designated the PIR2 protein by Toh-e *et al* (1993). The *HSP150/PIR2* gene was shown to be a member of a family of at least three genes *(PIR1*, *PIR2* and *PIR3*) all of which contain similar internal tandem repeats of approximately 19 amino acids. Homologues of the *PIR* genes were shown to be present also in *Kluyveromyces lactis* and *Zygosaccharomyces rouxii* (Toh-e *et al*, 1993). Disruption of the *HSP150/PIR2* gene showed that this is not an essential gene (Russo *et al*, 1992; Toh-e *et al*, 1993).

In this specification we refer to rHA as the desired protein. However, it is to be understood that the problem addressed by the invention will, in principle, be encountered with any other protein which has similar properties to those of rHA and which is therefore purified in the same way. Thus, the solution provided by the invention, namely elimination of Hsp150, is applicable also to the production of such other proteins.

Our studies have revealed that the Hsp150 protein is inefficiently separated from rHA by ion exchange chromatography. Surprisingly, however, Hsp150 does not appear in the fraction equivalent to the rHA fraction when rHA is absent. For example, when rHA-containing culture supernatant is passed through a cation exchange column under conditions which ensure binding of the rHA to the column (eg pH4.5, conductivity <7mS), Hsp150 also binds to the column and is eluted under the same conditions as rHA and thus contaminates

the rHA preparation. However, when culture supernatant from a yeast that does not secrete rHA is passed through such a column under the same conditions, the Hsp150 protein does not bind to the matrix but passes straight through the column. The eluate fraction does not contain Hsp150 in the absence of rHA. Similarly, the Hsp150 protein does not bind to an anion exchange column run under conditions which would result in binding of albumin (eg pH5.5, 1.5mS) in the absence of rHA, but is present in the rHA eluate fraction when rHA is present. Surprisingly, we have found that the presence of rHA in culture supernatant significantly alters the behaviour of some yeast proteins during chromatographic purification of the rHA such that proteins with physico-chemical properties which indicate that they would be separated from albumin by, for instance, ion exchange chromatography in fact contaminate the rHA preparation and are difficult to remove.

One aspect of the invention provides a process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).

The most convenient way of achieving this is to create a yeast which has a defect in its genome such that a reduced level of the Hsp150 protein is produced. For example, there may be a deletion, insertion or transposition in the coding sequence or the regulatory regions (or in another gene regulating the expression of the Hsp150 gene) such that little or no Hsp150 protein is produced. Alternatively, the yeast could be transformed to produce an anti-Hsp150 agent, such as an anti-Hsp150 antibody.

To modify the *HSP150* gene so that a reduced level of co-purifying protein is produced, site-directed mutagenesis or other known techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies

and Applications of *In Vitro* Mutagenesis", *Science*, **229**: 193-210 (1985), which is incorporated herein by reference. Suitable mutations include chain termination mutations (clearly stop codons introduced near the 3′ end might have insufficient effect on the gene product to be of benefit; the person skilled in the art will readily be able to create a mutation in, say, the 5′ three quarters of the coding sequence), point mutations that alter the reading frame, small to large deletions of coding sequence, mutations in the promoter or terminator that affect gene expression and mutations that de-stabilize the mRNA. Some desirable point mutations or specific amino acid substitutions may affect chromatographic behaviour by altering the charge distribution. Hence, the protein produced has a similar primary amino acid sequence to that of native Hsp150, but is functionally distinct such that it will not co-purify with the desired protein. Such a modified protein is not regarded as being Hsp150. Specific mutations can be introduced by an extension of the gene disruption technique known as gene transplacement (Winston, F. *et al* (1983) *Methods Enzymol.* **101**, 211-228).

Any polypeptides inserted into the Hsp150 protein should not be, and should not create, ligands for the desired protein. Those skilled in the art can readily determine, by simple binding assays, whether a ligand has been used or created. Generally one uses a selectable marker to disrupt a gene sequence, but this need not be the case, particularly if one can detect the disruption event phenotypically. In many instances the insertion of the intervening sequence will be such that a stop codon is present in frame with the Hsp150 sequence and the inserted coding sequence is not translated. Alternatively the inserted sequence may be in a different reading frame to Hsp150.

The gene may have one or more portions (optionally including regulatory regions, up to the whole gene) excised or inverted, or it may have a portion inserted, in order to result either in no production of protein from the *HSP150*

locus or in the production of protein from the *HSP150* locus which does not co-purify with the desired protein.

Preferably, the yeast secretes the desired protein, which is then purified from the fermentation medium. The purification may take place elsewhere; hence, production of culture medium, containing desired protein, in which the level of Hsp150 protein is low or zero is an end in itself.

A protein is generally regarded as co-purifying with Hsp150 if the two are still associated after two dissimilar chromatographic separation techniques (one of which is affinity chromatography for the desired protein) or, if affinity chromatography is not used, if the proteins are still associated after three dissimilar steps (for example an anion exchange, a cation exchange and a gel permeation step). Essentially, the identity of the desired protein is self-defined: if a person skilled in the art finds that his desired protein is, after an otherwise suitable purification process, contaminated with a yeast protein, he can determine (using known methods, which are explained in more detail below) whether that yeast protein is Hsp150 and, if it is, use the yeasts and methods of the invention; if the desired protein is not contaminated with Hsp150, then the need for the present invention will not arise. We have found the process of the invention to be particularly applicable to albumins and to other proteins which have similar physico-chemical properties to albumins, such that they are purified by similar chromatographic techniques. Preferably, the desired protein is a human albumin.

Human serum albumin (HSA) is a protein of 585 amino acids that is present in human serum at a concentration of 35-45g L^{-1} and represents about 60% of the total serum protein. HSA is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe

burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

The albumin may be a variant of normal HSA/rHA. By "variants" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin, for example those fragments disclosed in EP 322 094 (namely HSA (1-n), where n is 369 to 419); and fusions of albumin with other proteins, for example the kind disclosed in WO 90/13653.

By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

A second main aspect of the invention provides a yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in such Hsp150.

In addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

The desired protein is produced in conventional ways, for example from a coding sequence inserted in the yeast chromosome or on a free plasmid.

The yeasts are transformed with a coding sequence for the desired protein in any of the usual ways, for example electroporation. Methods for

transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct can be grown to produce the desired polypeptide. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies.

Useful yeast plasmid vectors include pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic

activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA in accordance with the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

Any yeast which produces an Hsp150 protein can be modified in accordance with the invention. Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia (Hansenula)*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschunikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like.

Preferred genera are those selected from the group consisting of *Saccharomyces, Schizosaccharomyces, Kluyveromyces,* and *Torulaspora.* Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii.* Examples of *Kluyveromyces* spp. are *K. fragilis*, *K. lactis* and *K. marxianus.* A suitable *Torulaspora* species is *T. delbrueckii.* Examples of *Pichia (Hansenula)* spp. are *P. angusta* (formerly *H. polymorpha*), *P. anomala* (formerly *H. anomala*) and *P. pastoris.*

Homologues of *HSP150* have already been shown to be present in a wide range of different yeast genera: *Torulaspora* sp., *Kluyveromyces* sp., *Schizosaccharomyces* sp. and *Zygosaccharomyces* sp. (Russo *et al*, 1992; Toh-e *et al*, 1993). In addition, our own studies have shown by Southern blotting that *Pichia* sp. possess a homologue of *HSP150.*

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, the *GPD1* promoter, and hybrid promoters involving hybrids of parts of 5′ regulatory regions with parts of 5′ regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are the thiamine-repressible promoter from the *nmt* gene as described by Maundrell

(1990) *J. Biol. Chem.* **265**, 10857-10864 and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990) *Genetics* **124**, 807-816.

Methods of transforming *Pichia* for expression of foreign genes are taught in, for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The desired protein may be initially expressed with a secretion leader sequence, which may be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae* include that from the mating factor α polypeptide (MFα-1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. Further such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-

sequence of MFα-1, β-glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α-galactosidase (*MEL1*); *K. lactis* killer toxin; and *Candida* glucoamylase.

Detailed Description of the Invention

Preferred aspects of the invention will now be described in more detail, with reference to the accompanying drawings, in which

Figure 1 is a scheme showing the preparation of an *Eco*RI *HSP150-URA3-HSP150* fragment used to transform a yeast strain (DBU3) and disrupt the *HSP150* gene (Example 1); and

Figure 2 is a scheme showing the preparation of a further *Eco*RI fragment used to remove the *HSP150* coding sequence altogether (Example 2).

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding rHA are derived from the cDNA disclosed in EP 201 239.

Example 1

The *HSP150* gene was mutated by the process of gene disruption (Rothstein, 1983) which effectively deleted part of the *HSP150* coding sequence, thereby preventing the production of Hsp150.

Four oligonucleotides suitable for the PCR amplification of the 5′ and 3′ ends of the *HSP150* gene (Russo *et al*, 1992) were synthesized using an Applied Biosystems 380B Oligonucleotide Synthesizer.

<u>5′ End</u>

LRE45: 5′-CTATTTCCTATTTCGGGAATTCTTAAAGACAAAAAAGCTC-3′

LRE46: 5′-GGCTGTGGTGCTGCAGATGATGCGCTGG-3′

<u>3′ End</u>

LRE47: 5′-GCTACTTCCGCTTCTGCAGCCGCTACCTCC-3′

LRE48: 5′-GCCGTGTAGCGAGGGAATTCTGTGGTCACGATCACTCG-3′

Note, LRE45 and LRE48 contain changes in the *HSP150* gene sequence so as to introduce *Eco*RI sites into the 5′ or the 3′ end of the *HSP150* gene PCR products. LRE46 and LRE47 both contain *Pst* I sites naturally present in the *HSP150* gene sequence (SEQ 1).

PCR was carried out to amplify individually the 5′ and 3′ ends of the *HSP150* gene, using LRE45 and LRE46 or LRE47 and LRE48 respectively, from the DNA from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc.).

Conditions were as follows: 1μg/ml genomic DNA, $\simeq 1.2\times10^{-10}$ moles of each primer, denature at 94°C for 61 seconds, anneal at 37°C for 121 seconds, DNA synthesis at 72°C for 181 seconds for 30 cycles, with a 10 second extension to the DNA synthesis step after each cycle, followed by a 4°C soak. PCR was carried out using a Perkin-Elmer-Cetus Thermal cycler and a Perkin-Elmer-Cetus PCR kit was used according to the manufacturer's recommendations. PCR products were analysed by gel electrophoresis and were found to be of the expected size. Each PCR product was digested with *Eco*RI and *Pst*I and cloned into *Eco*RI/*Pst*I digested pUC19 (Yanisch-Perron *et al,* 1985) to form pAYE503 (containing the 5′ end of the *HSP150* gene) and pAYE504 (containing the 3′ end of the *HSP150* gene) (see Fig. 1).

Plasmid DNA sequencing was carried out on pAYE503 and pAYE504 to

confirm that the inserts were the desired sequences. pAYE503 and pAYE504 were digested with *Eco*RI and *Hin*dIII and the *HSP150* gene fragments were isolated and cloned together into pUC19XH (a derivative of pUC19 lacking a *Hin*dIII site in its polylinker) to form pAYE505. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hin*dIII fragment and cloned into the *Hin*dIII site of pAYE505 to form pAYE506 (Fig. 1). pAYE506 contains a selectable marker (*URA3*) flanked by 5′ and 3′ regions of the *HSP150* gene.

To construct a strain lacking the capacity to produce HSP150, a *ura3* derivative of DB1 cir^o pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). Plasmid pAYE316 is based on the 2 μm plasmid and contains a coding sequence for human albumin under the control of the yeast *PRB1* promoter, with an *ADH1* terminator and a *LEU2* selectable marker.

The strain was grown overnight in 100mL buffered minimal medium (Yeast Nitrogen Base [without amino acids, without ammonium sulphate, Difco], $(NH_4)_2SO_4$ 5g/L, citric acid monohydrate 6.09g/L, $NaHPO_4$ 20.16g/L, sucrose 20g/L, pH6.5) and the cells were collected by centrifugation and then washed once with sterile water. The cells were then resuspended in 10mL sterile water and 2mL aliquots were placed in separate 15mL Falcon tubes. A 5mg/mL solution of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0μL, 20μL, 40μL, 80μL or 160μL. The cells were then incubated at 30°C for 20 min and then centrifuged and washed three times with sterile water. Finally, the cells were resuspended in 1mL YEP (1%$^w/_v$ yeast extract, 2%$^w/_v$ Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2%$^w/_v$ sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were grown on YEP plates containing 2%$^w/_v$ sucrose and then replica-plated onto

YNB minimal medium containing 2%$^{w}/_{v}$ sucrose and supplemented with 5-fluoro-orotic acid (1mg/mL) and uracil (50μg/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by introduction of the *URA3* gene by transformation.

The *ura3* strain, DBU3 cir° (pAYE316), was transformed with *Eco*RI digested pAYE506 and Ura^{+} transformants were selected. The disruption of the *HSP150* gene in these transformants was confirmed by Southern blot analysis using a fragment comprising the 5′ and 3′ ends of the *HSP150* gene (the *Eco*RI fragment from pAYE505) as a probe.

The yeast was then grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 ± 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH_2PO_4	114.0
$MgSO_4$	12.0
$CaCl_2.6H_2O$	3.0
Na_2EDTA	2.0

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
$ZnSO_4.7H_2O$	3.0
$FeSO_4.7H_2O$	10.0
$MnSO_4.4H_2O$	3.2
$CuSO_4.5H_2O$	0.079
H_3BO_3	1.5
KI	0.2
$Na_2MoO_4.2H_2O$	0.5
$CoCl_2.6H_2O$	0.56
H_3PO_4	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m-inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, $(NH_4)_2SO_4$ 5 g/L, citric acid monohydrate 6.09 g/L, Na_2HPO_4 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun, Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h^{-1}). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved.

The fermentation broth was centrifuged to remove the cells and then subjected to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0. The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

Analysis of rHA purified from *Hsp150* strains revealed that no HSP150 protein was present in these samples. HSP150 protein is determined using prior art techniques such as ELISA or Western blotting.

Anti-HSP150 antibodies are disclosed in Russo *et al* (1992) *Proc. Nat. Acad.*

Sci. (USA) **89**, 3671-3675.

Example 2

The *HSP150* protein coding sequence was deleted by using alternative fragments of the cloned *HSP150* sequences as follows.

The *URA3 Hin*dIII fragment from YEp24 (see Example 1) was cloned into pIC19R (Marsh J.L. *et al* (1984) *Gene* **32**, 481-485) at *Hin*dIII to form pAYE601 and then excised as a *SalI*/*Cla*I fragment and inserted into pAYE505 at the *Xho*I and *Cla*I sites to form pAYE602 (Fig 2). This plasmid was digested with *Eco*RI and then used to transform DBU3 cir° (pAYE316), selecting for Ura^{+} transformants. The disruption of the *HSP150* gene in these transformants was confirmed by Southern blot analysis as described in Example 1.

Thus, in this example, the whole of the *HSP150* coding sequence is removed, whereas in Example 1 the sequence is disrupted to yield non-functional protein.

Example 3

Southern blotting has revealed an Hsp150 homologue in *Hansenula polymorpha* (now called *Pichia angusta*). The *P. angusta* gene may be functionally deleted by ways analogous to those in Examples 1 and 2.

References

Boeke, J. D. *et al* (1987) *Methods Enzymol.* **154**, 164-175.

Botstein, D. *et al* (1979) *Gene* **8**, 17-24.

Collins, S.H. (1990) In Protein Production by Biotechnology (Harris, T.J.R., ed.) pp 61-77, Elsevier, Barking, Essex.

Curling (1980) "Albumin Purification by Ion Exchange Chromatography", in "Methods of Plasma Protein Purification", Ed. Curling, J.M., Academic Press, London.

Fleer, R. *et al* (1991) *Bio/Technology* **9**, 968-975.

Maurel *et al* (1989) "Biotechnology of Plasma Proteins", Colloque INSERM **175**, 19-24.

Romanos, M. *et al* (1992) *Yeast* **8**, 423-488.

Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202-211.

Russo, P. *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 3671-3675.

Sambrook, J. *et al* (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sleep, D. *et al* (1991) *Bio/Technology* **9**, 183-187.

Toh-e *et al* (1993) *Yeast* **9**, 481-494.

Wang, H.Y. *et al* (1979) *Biotechnology & Bioeng.* **21**, 975

Yanisch-Perron, C. *et al* (1985) *Gene* **33**, 103-119.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Delta Biotechnology Limited
(B) STREET: Castle Court, Castle Boulevard
(C) CITY: Nottingham
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast Strains

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9411356.0
(B) FILING DATE: 07-JUN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..40
(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of 5' end of Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTATTTCCTA TTTCGGGAAT TCTTAAAGAC AAAAAAGCTC
40

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..28
(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of the 5' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGCTGTGGTG CTGCAGATGA TGCGCTGG
28

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..30
(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of 3' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTACTTCCG CTTCTGCAGC CGCTACCTCC
30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..38
(D) OTHER INFORMATION: /note= "Oligonucleotide for PCR amplification of the 3' end of the Hsp150 gene."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCGTGTAGC GAGGGAATTC TGTGGTCACG ATCACTCG
38

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2048 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 397..1638

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGTGATCTTA CTATTTCCTA TTTCGGAAAT TATTAAAGAC
AAAAAAGCTC ATTAATGGCT 60

TTCCGTCTGT AGTGATAAGT CGCCAACTCA GCCTAATTTT
TCATTTCTTT ACCAGATCAG 120

GAAAACTAAT AGTACAAATG AGTGTTTTCT CAAGCGGAAC
ACCACATTTT GAGCTAAATT 180

TAGATTTTGG TCAAAATAAG AAAGATCCTA AAAAAGGAAT
GGTTGGTGAA AAATTTATTA 240

GCTTGAATGG TAGGAATCCT CGAGATATAA AAGGAACACT
TGAAGTCTAA CGACAATCAA 300

TTTCGATTAT GTCCTTCCTT TTACCTCAAA GCTCAAAAAA
ATATCAATAA GAAACTCATA 360

TTCCTTTTCT AACCCTAGTA CAATAATAAT AATATA ATG CAA
TAC AAA AAG ACT 414
Met Gln Tyr Lys Lys Thr
1 5

TTG GTT GCC TCT GCT TTG GCC GCT ACT ACA TTG GCC GCC
TAT GCT CCA 462
Leu Val Ala Ser Ala Leu Ala Ala Thr Thr Leu Ala Ala Tyr Ala Pro
10 15 20

TCT GAG CCT TGG TCC ACT TTG ACT CCA ACA GCC ACT TAC
AGC GGT GGT 510

Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr Ala Thr Tyr Ser Gly Gly
25 30 35

GTT ACC GAC TAC GCT TCC ACC TTC GGT ATT GCC GTT CAA
CCA ATC TCC 558
Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile Ala Val Gln Pro Ile Ser
40 45 50

ACT ACA TCC AGC GCA TCA TCT GCA GCC ACC ACA GCC TCA
TCT AAG GCC 606
Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr Thr Ala Ser Ser Lys Ala
55 60 65 70

AAG AGA GCT GCT TCC CAA ATT GGT GAT GGT CAA GTC CAA
GCT GCT ACC 654
Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Ala Thr
75 80 85

ACT ACT GCT TCT GTC TCT ACC AAG AGT ACC GCT GCC GCC
GTT TCT CAG 702
Thr Thr Ala Ser Val Ser Thr Lys Ser Thr Ala Ala Ala Val Ser Gln
90 95 100

ATC GGT GAT GGT CAA ATC CAA GCT ACT ACT AAG ACT ACC
GCT GCT GCT 750
Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala
105 110 115

GTC TCT CAA ATT GGT GAT GGT CAA ATT CAA GCT ACC ACC
AAG ACT ACC 798
Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Lys Thr Thr
120 125 130

TCT GCT AAG ACT ACC GCC GCT GCC GTT TCT CAA ATC AGT
GAT GGT CAA 846
Ser Ala Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Ser Asp Gly Gln
135 140 145 150

ATC CAA GCT ACC ACC ACT ACT TTA GCC CCA AAG AGC ACC
GCT GCT GCC 894
Ile Gln Ala Thr Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala
155 160 165

GTT TCT CAA ATC GGT GAT GGT CAA GTT CAA GCT ACC ACC

ACT ACT TTA 942
Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Thr Thr Leu
170 175 180

GCC CCA AAG AGC ACC GCT GCT GCC GTT TCT CAA ATC GGT
GAT GGT CAA 990
Ala Pro Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln
185 190 195

GTT CAA GCT ACT ACT AAG ACT ACC GCT GCT GCT GTC TTT
CAA ATT GGT 1038
Val Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Phe Gln Ile Gly
200 205 210

GAT GGT CAA GTT CTT GCT ACC ACC AAG ACT ACT CGT GCC
GCC GTT TCT 1086
Asp Gly Gln Val Leu Ala Thr Thr Lys Thr Thr Arg Ala Ala Val Ser
215 220 225 230

CAA ATC GGT GAT GGT CAA GTT CAA GCT ACT ACC AAG ACT
ACC GCT GCT 1134
Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
235 240 245

GCT GTC TCT CAA ATC GGT GAT GGT CAA GTT CAA GCA ACT
ACC AAA ACC 1182
Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr
250 255 260

ACT GCC GCA GCT GTT TCC CAA ATT ACT GAC GGT CAA GTT
CAA GCC ACT 1230
Thr Ala Ala Ala Val Ser Gln Ile Thr Asp Gly Gln Val Gln Ala Thr
265 270 275

ACA AAA ACC ACT CAA GCA GCC AGC CAA GTA AGC GAT GGC
CAA GTC CAA 1278
Thr Lys Thr Thr Gln Ala Ala Ser Gln Val Ser Asp Gly Gln Val Gln
280 285 290

GCT ACT ACT GCT ACT TCC GCT TCT GCA GCC GCT ACC TCC
ACT GAC CCA 1326
Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala Ala Thr Ser Thr Asp Pro
295 300 305 310

GTC GAT GCT GTC TCC TGT AAG ACT TCT GGT ACC TTA GAA ATG AAC TTA 1374
Val Asp Ala Val Ser Cys Lys Thr Ser Gly Thr Leu Glu Met Asn Leu
315 320 325

AAG GGC GGT ATC TTA ACT GAC GGT AAG GGT AGA ATT GGT TCT ATT GTT 1422
Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly Arg Ile Gly Ser Ile Val
330 335 340

GCT AAC AGA CAA TTC CAA TTT GAC GGT CCA CCA CCA CAA GCT GGT GCC 1470
Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro Pro Pro Gln Ala Gly Ala
345 350 355

ATC TAC GCT GCT GGT TGG TCT ATA ACT CCA GAC GGT AAC TTG GCT ATT 1518
Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro Asp Gly Asn Leu Ala Ile
360 365 370

GGT GAC AAT GAT GTC TTC TAC CAA TGT TTG TCC GGT ACT TTC TAC AAC 1566
Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu Ser Gly Thr Phe Tyr Asn
375 380 385 390

TTG TAC GAC GAA CAC ATT GGT AGT CAA TGT ACT CCA GTC CAC TTG GAA 1614
Leu Tyr Asp Glu His Ile Gly Ser Gln Cys Thr Pro Val His Leu Glu
395 400 405

GCT ATC GAT TTG ATA GAC TGT TAAGCAGAAA ACTATTAGTT CTTTTATCCT 1665
Ala Ile Asp Leu Ile Asp Cys
410

GATGACTTTT TCTCATTTGC ATTGATTAGA AAGGAAAAAA AGAAGTGTCC TTTTCTACTA 1725

CTACTCTAGT CGCATCCATT CCTTTGCATT TATCTTTTCT GCGGTTGGCC AATCCATTCT 1785

TCCGAGAATT TGGCTAGCCA TACTTGATGT TTTCCCATTA TTGGTTCGTT TGGCAATGCT 1845

AATTTTCTTA ATTGCCCCTT ATATACTCTT CCATAAAATG TTTTTTTTAT AACTAATTTT 1905

CTGTATATCA TTATCTAATA ATCTTATAAA ATGTTAAAAA GACTTGGAAA GCAACGAGTG 1965

ATCGTGACCA CATAATTGCC TCGCTACACG GCAAAAATAA GCCAGTCCTA ATGTGTATAT 2025

TAAAGGCTGC ATGTGGCTAC GTC
2048

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 413 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Gln Tyr Lys Lys Thr Leu Val Ala Ser Ala Leu Ala Ala Thr Thr
1 5 10 15

Leu Ala Ala Tyr Ala Pro Ser Glu Pro Trp Ser Thr Leu Thr Pro Thr
20 25 30

Ala Thr Tyr Ser Gly Gly Val Thr Asp Tyr Ala Ser Thr Phe Gly Ile
35 40 45

Ala Val Gln Pro Ile Ser Thr Thr Ser Ser Ala Ser Ser Ala Ala Thr
50 55 60

Thr Ala Ser Ser Lys Ala Lys Arg Ala Ala Ser Gln Ile Gly Asp Gly
65 70 75 80

Gln Val Gln Ala Ala Thr Thr Thr Ala Ser Val Ser Thr Lys Ser Thr
85 90 95

Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr
100 105 110

Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Ile Gln
115 120 125

Ala Thr Thr Lys Thr Thr Ser Ala Lys Thr Thr Ala Ala Ala Val Ser
130 135 140

Gln Ile Ser Asp Gly Gln Ile Gln Ala Thr Thr Thr Thr Leu Ala Pro
145 150 155 160

Lys Ser Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln
165 170 175

Ala Thr Thr Thr Thr Leu Ala Pro Lys Ser Thr Ala Ala Ala Val Ser
180 185 190

Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Ala Ala
195 200 205

Ala Val Phe Gln Ile Gly Asp Gly Gln Val Leu Ala Thr Thr Lys Thr
210 215 220

Thr Arg Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr
225 230 235 240

Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Gly Asp Gly Gln Val
245 250 255

Gln Ala Thr Thr Lys Thr Thr Ala Ala Ala Val Ser Gln Ile Thr Asp
260 265 270

Gly Gln Val Gln Ala Thr Thr Lys Thr Thr Gln Ala Ala Ser Gln Val
275 280 285

Ser Asp Gly Gln Val Gln Ala Thr Thr Ala Thr Ser Ala Ser Ala Ala
290 295 300

Ala Thr Ser Thr Asp Pro Val Asp Ala Val Ser Cys Lys Thr Ser Gly
305 310 315 320

Thr Leu Glu Met Asn Leu Lys Gly Gly Ile Leu Thr Asp Gly Lys Gly
325 330 335

Arg Ile Gly Ser Ile Val Ala Asn Arg Gln Phe Gln Phe Asp Gly Pro
340 345 350

Pro Pro Gln Ala Gly Ala Ile Tyr Ala Ala Gly Trp Ser Ile Thr Pro
355 360 365

Asp Gly Asn Leu Ala Ile Gly Asp Asn Asp Val Phe Tyr Gln Cys Leu
370 375 380

Ser Gly Thr Phe Tyr Asn Leu Tyr Asp Glu His Ile Gly Ser Gln Cys
385 390 395 400

Thr Pro Val His Leu Glu Ala Ile Asp Leu Ile Asp Cys
405 410

<u>CLAIMS</u>

1. A process for preparing a desired protein from yeast, comprising culturing the yeast and obtaining the desired protein, characterised in that the yeast is deficient in heat shock protein 150 (Hsp150).

2. A process according to Claim 1 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.

3. A process according to Claim 2 wherein substantially no Hsp150 protein is produced.

4. A process according to any one of the preceding claims wherein the desired protein is an albumin.

5. A process according to Claim 4 wherein the desired protein is a human albumin.

6. A process according to any one of the preceding claims wherein the yeast is a *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces*, *Pichia* or *Saccharomyces* species.

7. A process according to Claim 6 wherein the yeast is *S. cerevisiae*.

8. A process according to any one of the preceding claims wherein the desired protein is secreted from the yeast into the surrounding medium and purified therefrom.

9. A protein prepared by a process according to any one of the preceding claims.

10. A culture medium containing a desired protein and prepared by a process according to any one of Claims 1 to 7.

11. A yeast transformed to express a desired protein which will co-purify with Hsp150 in chromatographic techniques, characterised in that the yeast is deficient in Hsp150.

12. A yeast according to Claim 11 wherein the yeast has a defect in its genome such that a reduced level of the Hsp150 protein is produced.

13. A yeast according to Claim 11 wherein substantially no Hsp150 protein is produced by the yeast.

14. A yeast according to any one of Claims 11 to 13 wherein the desired protein is an albumin.

15. A yeast according to Claim 14 wherein the desired protein is a human albumin.

16. A yeast according to any one of Claims 11 to 15 wherein the yeast is *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces* or *Saccharomyces* species.

17. A yeast according to Claim 16 wherein the yeast is *S. cerevisiae*.

18. A yeast according to any one of Claims 11 to 17 wherein the yeast is transformed with a DNA construct such that the desired protein is secreted from the yeast during culturing thereof.

19. A method of preparing a yeast according to any one of Claims 11 to 18

comprising the steps of

(i) transforming the yeast with a coding sequence for expression of the desired protein, and

(ii) disrupting the genome of the yeast such that the yeast has an abnormally low level of Hsp150,

wherein steps (i) and (ii) may be carried out in either order or simultaneously.

5' and 3' regions of *HSP150* gene obtained by PCR:

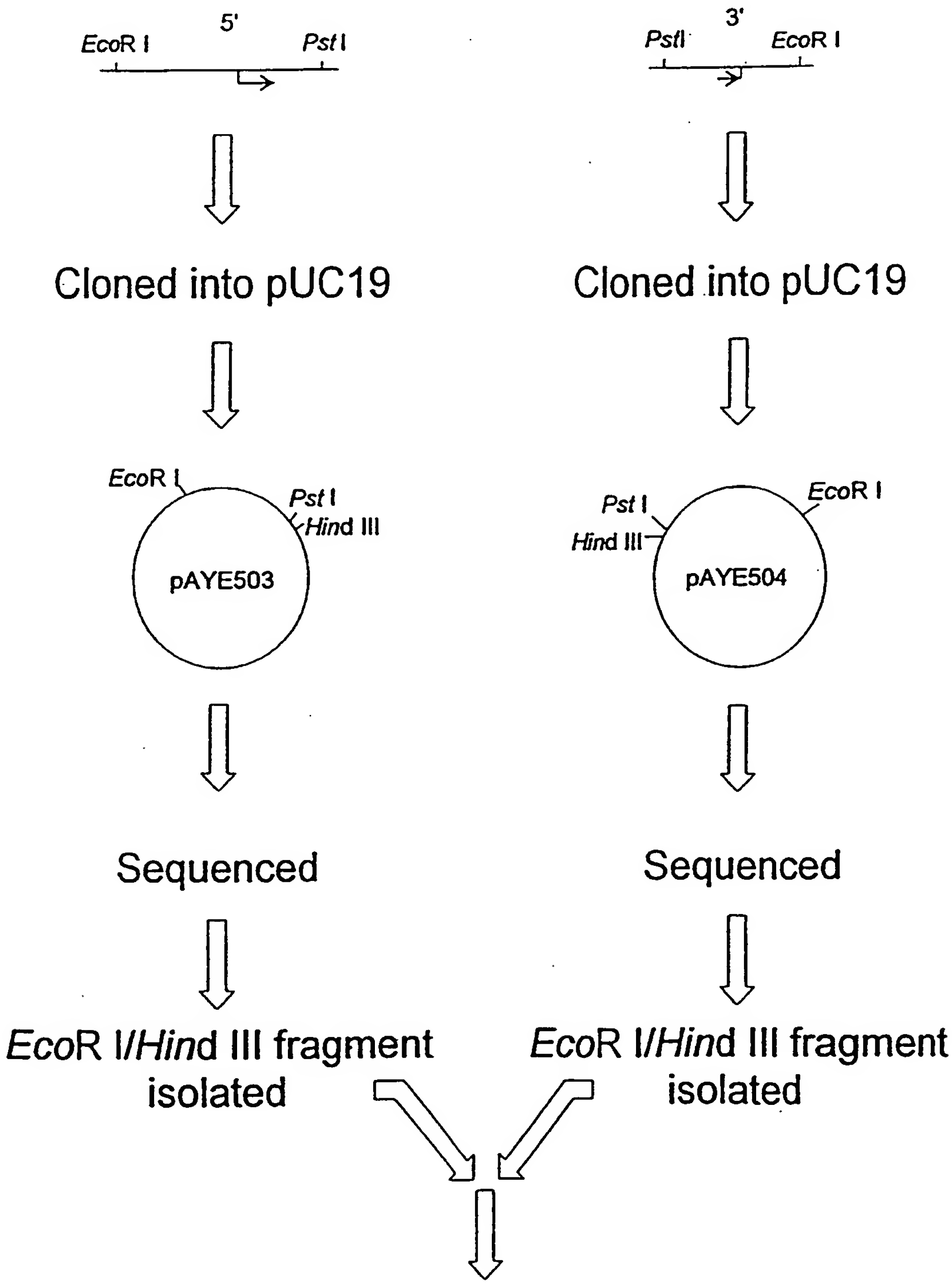


Figure 1

Figure 1 cont'd

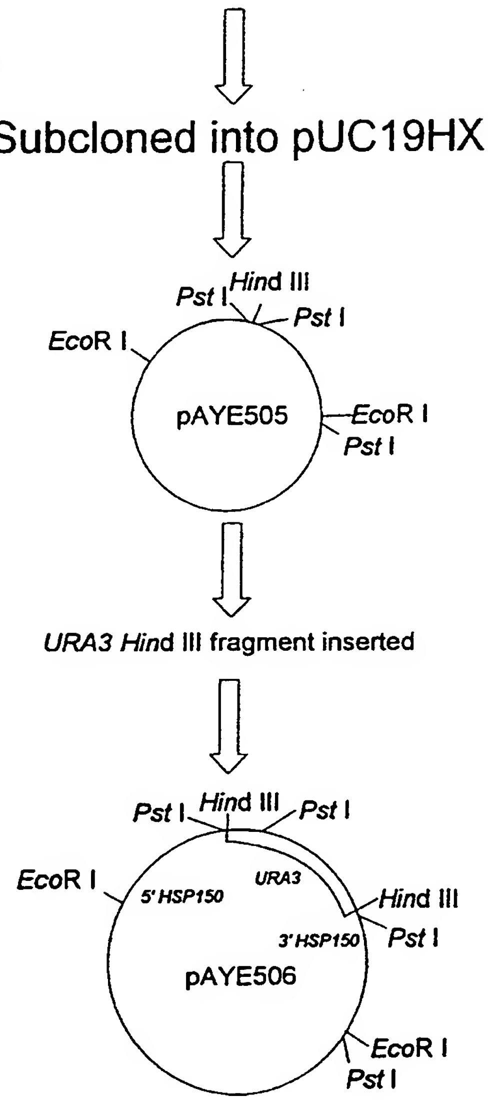
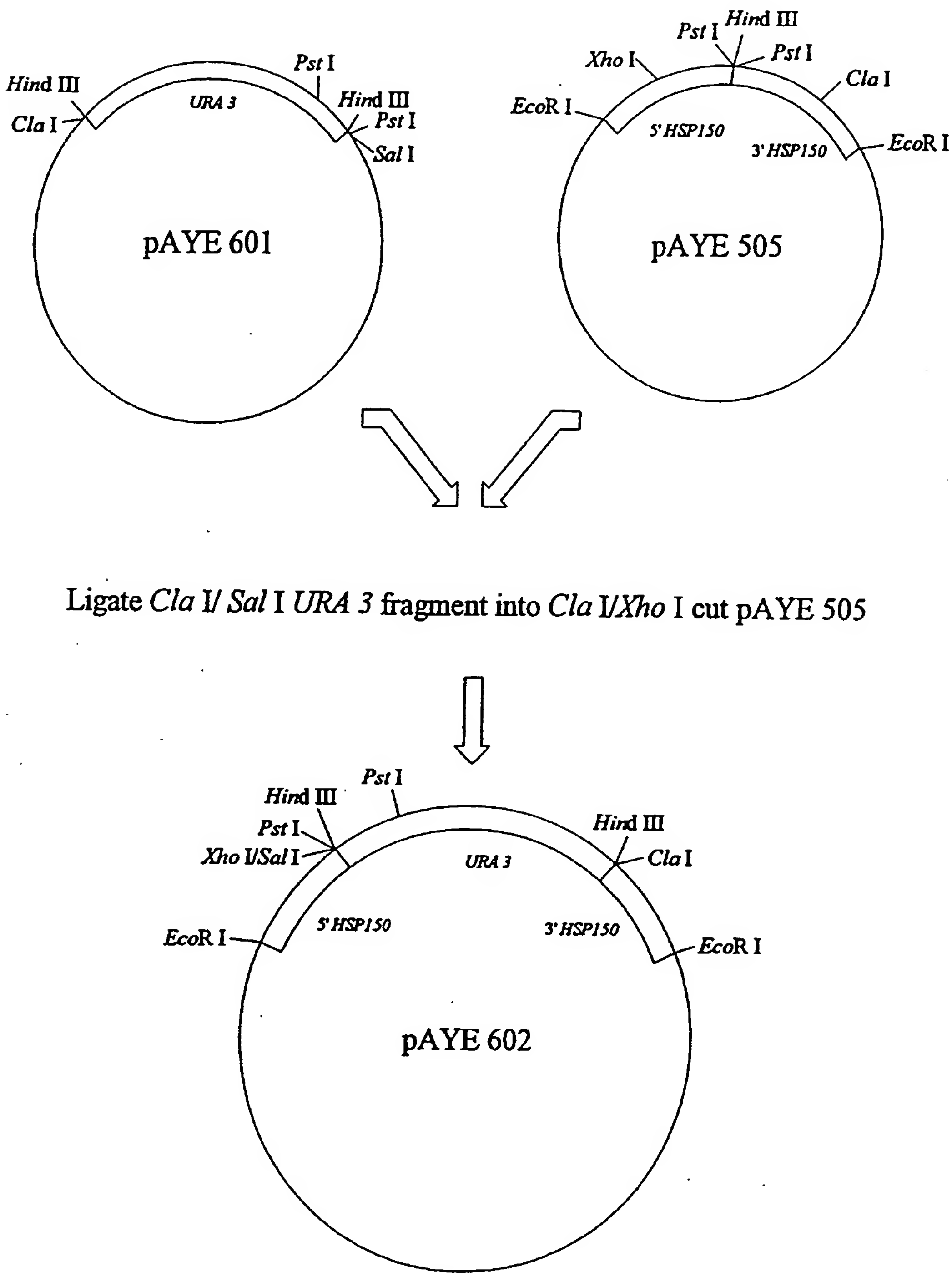


Fig 2


Hind III
Cla I
URA 3
Pst I
Hind III
Pst I
Sal I
pAYE 601
Pst I
Hind III
Xho I
Pst I
Cla I
EcoR I
5' HSP150
3' HSP150
EcoR I
pAYE 505
Ligate Cla I/ Sal I URA 3 fragment into Cla I/Xho I cut pAYE 505
Pst I
Hind III
Pst I
Xho I/Sal I
URA 3
Hind III
Cla I
EcoR I
5' HSP150
3' HSP150
EcoR I
pAYE 602

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB 95/01317

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/14 C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOL GEN GENET, MAY 1993, 239 (1-2) P273-80, GERMANY, RUSSO P ET AL 'Dual regulation by heat and nutrient stress of the yeast HSP150 gene encoding a secretory glycoprotein.' see the whole document ---	1,2, 6-12, 16-19
X	BIOTECHNOLOGY, vol. 8, 1990 NEW YORK US, pages 42-46, SLEEP D. ET AL. 'The secretion of human serum albumin from the yeast Sachcharomyces cerevisiae using five different leader sequences' cited in the application see the whole document -----	9,10

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1995	10.10.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J